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TELOFIT - Interaction between Physical Activity and Cellular Aging

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Graz, 30.04.2020

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// Zusammenfassung

Hintergrund und Ziele

Der aktuelle Wissensstand über den Einfluss von sportlicher Aktivität auf Zellalterung ist inhomogen und kontrovers. Besonders Telomere - die Chromosomenenden - werden in aktuellen Forschungen untersucht. Viele Faktoren und Mechanismen werden durch regelmäßiges sportliches Training beeinflusst und können dadurch auch einen Einfluss auf die Telomerfunktion haben. Manche Studien postulieren einen positiven und manchen keinen Zusammenhang zwischen regelmäßigen Sport und Zellalterung. Das Ziel dieser Studie ist es den Effekt von wiederholtem Sport auf die relative Telomerlänge und die Expression des Shelterin-Protein TERF-1 als Indikatoren für die Zellalterung zu untersuchen.

Methoden

Fünf Teilnehmerinnen (gesunde Frauen mittleren Alters) haben regelmäßige Fahrradergometer-Einheiten in verschiedenen Belastungsstufen über eine Zeit von drei Monaten absolviert. Die relative Telomerlänge und die Quantität von TERF-1 sind vor, während und nach den Blockeinheiten untersucht worden. Die relative Telomerlänge ist in peripheren Leukozyten mit einer quantitativen real-time PCR gemessen worden. TERF-1 ist mit einem kommerziellen ELISA gemessen worden.

Ergebnisse

Die Ergebnisse zeigen keine Assoziationen zwischen sportlicher Aktivität und relativer Telomerlänge oder TERF-1 Expression. Es sind auch keine Tendenzen in den Grafiken erkennbar, die einen direkten Zusammenhang vermuten lassen. Es ist allerdings eine statistisch signifikante Korrelation zwischen relativer Telomerlänge und TERF-1 Expression beobachtbar ($r=-0{,}49$; $p<0{,}01$).

Konklusion

Die erhaltenen Daten zeigen keine statistisch signifikanten Effekte von sportlicher Aktivität auf die relative Telomerlänge oder TERF-1 Expression. Die Korrelation zwischen relativer Telomerlänge und TERF-1 Expression zeigt, dass das Studiendesign geeignet für die Untersuchung der Fragestellung ist und es sich um

robuste Daten handelt. Es ist festzuhalten, dass die aktuelle Studie eine Pilotstudie mit einer kleinen Anzahl von Teilnehmerinnen ist. Rekapitulierend ist zu sagen, dass weitere Studien mit mehr TeilnehmerInnen notwendig sind, um einen potenziellen Einfluss von regelmäßigem Training auf die Telomerfunktion zu evaluieren.

Abstract

Background and Aims

The current standard of knowledge about the impact of physical activity on cell aging is inhomogeneous and controversial. In this context, telomeres - the chromosomal ends - are surveyed in current research. Many factors and mechanisms caused by regular exercise may influence telomere function. Some findings postulate a positive and some no correlation between continuous activity and cell aging. The target of this study is to investigate the effect of repeated sports on relative telomere length and the expression of the shelterin protein TERF-1 as indicators of cell aging.

Methods

Five participants (healthy and middle-aged females) took part in regular bicycle ergometer training sessions at different intensity levels over three months. The relative telomere length and the quantity of TERF-1 were surveyed before, during and after the intervention blocks. The relative telomere length was measured in peripheral leucocytes by a quantitative real-time PCR. The TERF-1 was measured by a commercial ELISA.

Results

The findings show no association between physical activity and relative telomere length or TERF-1 expression. No tendencies, which may indicate an association, are remarkable in the charts. However, a statistically significant correlation between relative telomere length and TERF-1 expression is observed ($r=-0{,}49$; $p<0{,}01$).

Conclusion

The obtained data show no statistically significant effects of physical activity on relative telomere length or expression of TERF-1. The correlation of relative telomere length and TERF-1 expression shows the suitability of the study design and robustness of the analyses. However, it must be noticed that the present study is a pilot study with currently only a small number of participants. Taken together, further investigations with more participants are necessary to evaluate a potential impact of regular exercise on telomere function.

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List of Abbreviations

Abbreviations	Meaning
A	Adenine
BMI	Body mass index
C	Cytosine
DNA	Deoxyribonucleic acid
eNOS	Endothelial nitric oxide synthase
FISH	Fluorescent in situ hybridisation
G	Guanine
hTERT	Human telomerase reverse transcriptase
LTL	Leucocyte telomere length
Max	Maximum
MET	Metabolic equivalents of task
Min	Minimum
MVPA	Moderate-to-vigorous physical activity
NO	Nitric oxide
PA	Physical activity
PCR	Polymerase chain reaction
POT1	Protector of telomeres 1
RAP1	Repressor/Activator protein 1
RTL	Relative telomere length
SD	Standard deviation
T	Thymine
T2D	Type 2 diabetes
TERC	Telomerase RNA component
TERF-1	Telomeric repeat binding factor 1
TERF-2	Telomeric repeat binding factor 2
TIN2	TERF-1 interacting nuclear factor 2
TL	Telomere length
TPP1	POT1-TIN2 organising protein

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1 Introduction

1.1 Telomere Biology

1.1.1 Deoxyribonucleic Acid

The human genetic information is stored in deoxyribonucleic acid (DNA) which is a molecule composed of two strands. It has been first discovered and described by Watson and Crick (1). The two strands are coiling around and forming a double helix. These DNA chains are constructed each by various aligned nucleotides and run anti-parallelly. Every nucleotide is composed of three parts – a base, a sugar and a phosphate group. Each nucleotide comprises of one of four bases which are divided into two groups. One group is called the purines, including the base guanine [G] and the base adenine [A] and the others are the pyrimidines, including the base cytosine [C] and the base thymine [T] that are linked at the C1′-end of the sugar. This sugar molecule presents the second component and is deoxyribose. The third part is a phosphate group which is linked at the sugar of one nucleotide and the following one. Nucleotides are connected to another in a row by bonds between the phosphate of one nucleotide and the sugar of the following. The antiparallel structure is stabilised by hydrogen bonds between the two strands. These bonds link directly between the bases and build the base pairs: Adenine pairs with thymine and cytosine pairs with guanosine. Thus, the DNA strands end asymmetrically with a directionality of five prime end (5′) and three prime end (3′). These different prime ends are caused by the links between the phosphate group and the deoxyribose. The 3′ end is completed by a terminal hydroxyl group and the 5′ end by a terminal phosphate group. The structure of the DNA is shown schematically in Figure 1 (2).

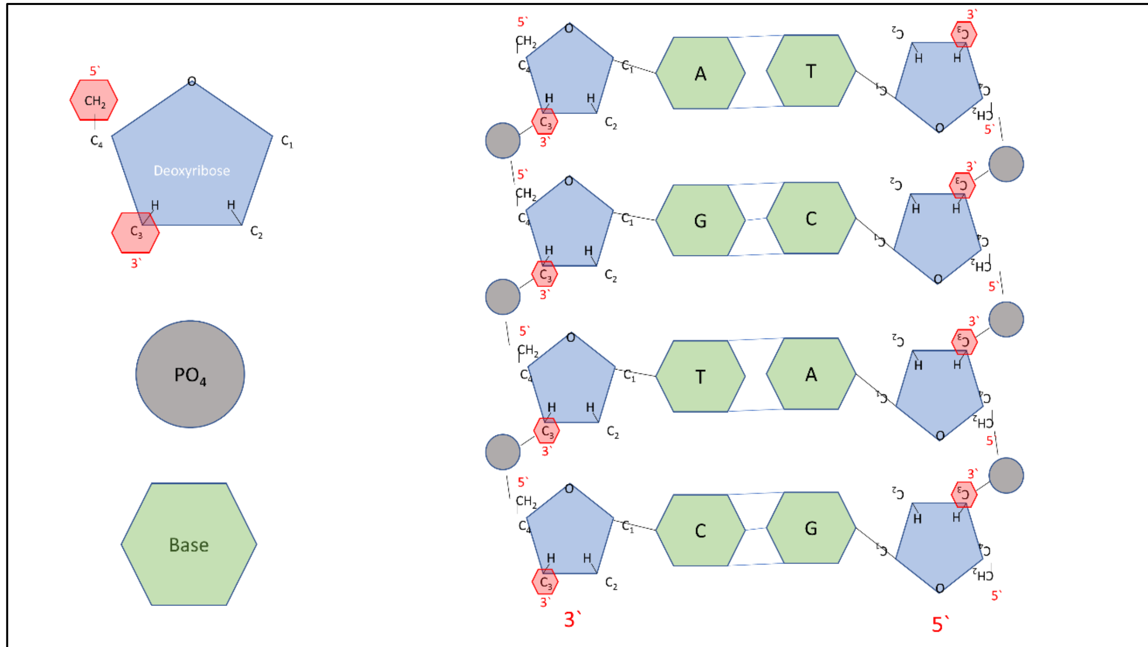

Figure 1: Structure of Deoxyribonucleic Acid
DNA is shown schematically; green hexagon represents one of the four bases; grey circle represents phosphate group; blue pentagon represents deoxyribose with red highlighted 3' and 5' end; base pairs are linked together by hydrogen bonds (blue lines); deoxyribose is linked with the following deoxyribose by phosphate groups; Source: own research

1.1.2 Telomeres

The chromosome ends are designated as telomeres and ensure genetic stability. At the beginning of the last century, the current standard of knowledge has believed that all vertebrate cells divide indefinitely in culture and seem to be immortal (3). The discovery of the Hayflick limit has been revolutionary at that time. It has propagated a defined amount of cell replications before cell division ends. In the early sixties of the twentieth century, this phenomenon has been investigated by the anatomist Leonard Hayflick with his colleagues. Hayflick has shown that cultured fibroblasts will divide between 50 ± 10 times. He has defined three phases in the life of cells in a cultured medium. At the beginning of his trial, he has named the primary state "phase one". The proliferation of cells has been defined as “phase two”. After a certain time of doubling, the population may reach “phase three”, the so-called "senescence". In this phase, the cell replication rate starts slowing down until it stops (4-6).

In the seventies of the last century, Blackburn and her colleague Gall have investigated extrachromosomal genes coding for ribosomal DNA in the ciliated protozoan *tetrahymena thermophila*. After the Hayflick limit had been currently discovered, the emphasis of genetic research has been focused on the cell division and its operating. Blackburn and Gall have been interested in the chromosome ends. They found a repetitive hexanucleotide sequence $(C-C-C-C-A-A)_n$ (*n = 20-70*) at the chromosomal ends. Defining this sequence has been one of the milestones in the history of research in telomeres (7, 8).

The name of the nucleoprotein telomere referrers to the ancient Greek words “telos” (end) and “meros” (part). Telomeres are located at each end of the chromosomes and compound by a repetitive, non-coding DNA sequence (see figure 2). They are surrounded by various telomeric interacting proteins. As described first by Moyzis

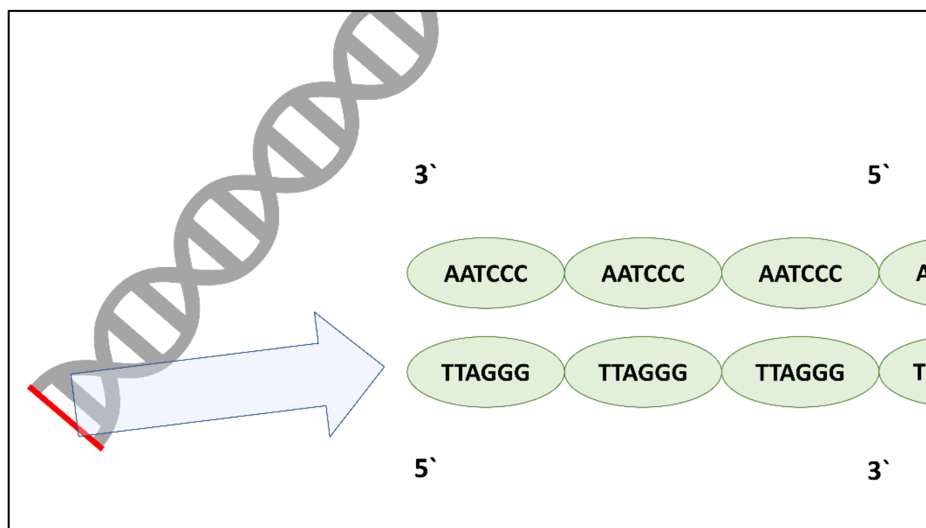

Figure 2: Chromosomal End
The figure shows schematically the end of the chromosomes and base sequence of telomeres; Source: own research

et al., these nucleoproteins extend numerous kilobase pairs and are composed of thousands of repeats of the hexanucleotide $(T-T-A-G-G-G)_n$ in humans. Telomeres obtain two essential purposes. On one side, they secure the chromosome ends by avoiding unintentional degradation and recombination of DNA. On the other side, they ensure the total coding without loss while DNA replication. Due to telomeres three-dimensional folding, it safeguards the 3′-end of the strand by avoiding being identified as a double-strand break. Further, the DNA

damage response is triggered when telomeres are unfunctional. In case of the absence of functional telomeres, there would be an unavoidable loss of genetic information with every cell division. Due to the fact that telomeres are non-coding sequences – containing no genetic information – and composed by repetitive base pairs, its shortening ensures the preservation of coding DNA sequences and genetic information. Consequently, somatic cells can only pass a few numbers of mitosis. In the case that telomeres reach a critical length of about 4000 base pairs, the cell passes in senescence or apoptosis. Thus, telomeres play a decisive role in the life span of cells. Avoiding an uncontrolled division of cells with critically short telomere length, these nucleoproteins constitute a valuable tumor suppressor structure. If the dividing is not stopped, like in some cancer cells, the whole chromosome and hence the cell itself become unstable and apoptosis is induced (8-10).

1.1.3 Telomerase

Telomerase is a ribonucleoprotein complex that harbours a reverse transcription activity and comprises of two parts. First, the well-conserved reverse transcriptase (human telomerase reverse transcriptase, hTERT). Second, the telomerase RNA component (TERC) that is used as a pattern for telomere prolongation by adding TTAGGG repetitions onto chromosome ends. TERC expression is omnipresent. Contrary, hTERT is mostly detectable for instance in germ cells, haemopoietic stem cells and keratinocytes in the basal layer of the epidermis but barely detectable in somatic cells. Further, some cancers show an elevated telomerase activity. Additionally, a failed telomerase activity seems to play an important role in the pathogenesis of many diseases like ataxia telangiectasia, heart failure and immunosenescence. Investigations in rodents showed shortened telomeres, unstable genomes, aneuploidy and age-related phenotypes after the deletion of either hTERT or TERC. Contrary, it was shown in mice that overexpression of TERT increases the life span of these rodents (8, 10, 11).

1.1.4 Shelterin

A complex formed by six telomere-specific proteins surrounds the telomeres. This complex is named Shelterin or telosome and its forming protects telomeres from degradation. Otherwise, chromosomal ends would be incorrectly edited by DNA repair mechanisms and degraded. The telosome is composed of Telomeric Repeat Binding Factor 1 and 2 (TERF-1 & -2), TERF-1 Interacting Nuclear Factor 2 (TIN2), Repressor/Activator Protein 1 (RAP1), Protector of Telomeres 1 (POT1) and POT1-TIN2 Organising Protein (TPP1). The telosome and its location is schematically sketched out in figure 3 (10, 12).

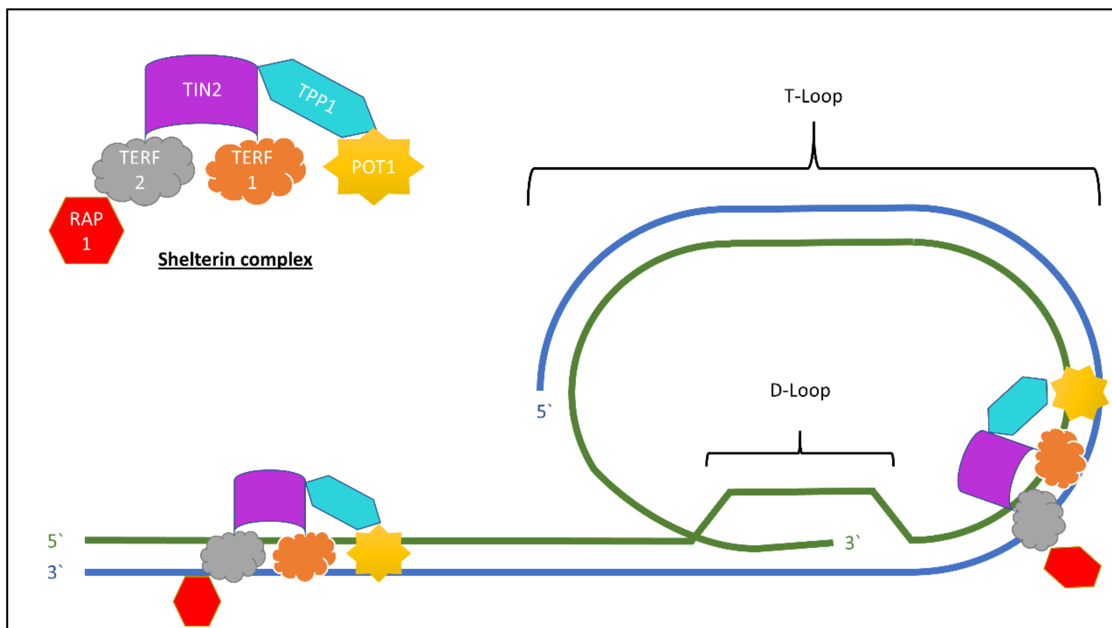

Figure 3: Shelterin Complex
The components of the Shelterin complex (TERF-1 & -2, TIN2, RAP1, POT1 and TPP1) are schematically shown top left; the two complementary strands of DNA are presented in green and blue; the formed t- and d- loop are indicated by braces; Source: own research

TERF-1 & -2 and POT1 identify TTAGGG repeats and bind to it. The function of TIN2 is to interconnect the parts of the shelterin complex by attaching to TERF-1 & -2, TPP1 and POT1. Additionally, TPP1 interacts with POT1 and the telomerase. RAP1 is attached to telomeric DNA with its connection to TERF-2. Further, RAP1 guards from recombination and capping of the telomeres. Recent research shows that telosomes are dynamic elements of the chromosomal ends that are mobilised while DNA remodelling processes. This is conducted by interacting with the

structure of the telomeres. The 3′ single-stranded DNA overhang at the end of the telomeres can fold back and connect with the double-stranded telomeric DNA, creating a loop structure. This part is named the T-loop. Thus, the free 3′ end of the strand is hidden by the closed configuration of the T-loop and escapes being recognised as a double-strand break interacting with the contrary strand named the D-loop. In this way, a shielding cap is constructed that is defining the end of the telomeres. Nevertheless, in somatic cells in every division, a loss of 50-200 base pairs of telomeric DNA must be assumed. Therefore, the telomeres are shortening continuously (8, 10, 12, 13).

1.2 Physical Activity and Telomere Biology

A central component of healthy aging is practicing regularly leisure sports (14). Recent guidelines recommend exercising more than 2,5 hours a week of moderate-to-vigorous physical activity to gain significant health benefits (15). The benefits of regular physical activity are important for the individual health status and therefore also for global health care systems. It is estimated that physical inactivity generates costs in the health-care systems worldwide of around 53,8 billion US Dollar (16). For these reasons, physical activity has been focused scientifically more intensive in the last decades. Physical activity habits are surveyed mainly by physical activity questionnaires (e.g. Minnesota Leisure-Time Activities Questionnaire), physical fitness measures like maximal oxygen consumption or metabolic equivalents of task (MET). The MET is measured objectively by the ratio of energy expenditure for sitting gently to the performed activity. The unit of MET is 3,5 ml of oxygen per kilogram per minute or calorie per kilogram per hour. Further in some investigations, groups of athletes and non-athletes are formed to determine a training and a control cohort. To summarise, the diverse generated datasets are complicating the investigations. This is also discussed in further reviews and meta-analysis (17-21).

Table 2 in the appendix gives a short overview of some investigations analysing physical activity and telomere length. As shown in the table, the study cohorts differ a lot. Some interventional studies comprise 20 participants, other surveys rely on

over 2000 probands. This may lead to a bias concerning the validity of the results. Because of the small number of participants, the collected data may not offer some significant outcomes and it can't be extrapolated from these findings to the entire population. Further, the methods of the surveys and the interventions are quite inequal and distinguish in many forms. For instance, one part is evaluated by self-reported questionnaires, whereas other studies are assessed by pedometer measurements or metabolic equivalent of task (MET). Especially the validity of self-reported questionnaires has to be reconsidered. With respect to these circumstances, comparability is not ensured at all. Moreover, findings are at least in part controversial due to unique analyses methods (e.g. PCR, Q-FISH, Southern blot) and diverse quantification methods (relative vs. absolute telomere length). While traditional methods like Southern blotting require the whole genomic DNA extraction for determining telomere length and do not allow to estimate the telomere length in individual cells, qPCR and Fish are more precise and faster methods (22, 23). Considering all these aspects, a widespread and heterogenous outcome is observable.

The pathways of physical activity influencing cellular senescence have been described variously in common literacy. Exercising may stimulate the upregulation of telomerase reverse transcriptase. Thus, it might sustain telomere length or support telomere lengthening. Further, physical activity activates the expression of insulin-like growth factor 1. Some investigations have exposed that low circulating levels of IGF-1 relate to shorter LTL comparing to higher levels of circulating IGF-1 (24). Further, it has been shown that habitual exercise impacts telomere length by reducing oxidative stress and inflammatory reactions. Telomere attrition is averted by anti-inflammatory and anti-oxidative reactions. Common approaches concerning anti-oxidative reactions have a positive effect on telomere length caused by decreased interleukin-6, tumor necrosis factor α and C-reactive protein (25, 26). Additionally, an increased bioavailability of endothelial NO (Nitric oxide) induced by exercise has been reported. In contrast, a decreased level of endothelial NO seems to be linked with aging processes. Current investigations show that the enzyme nitric oxide synthase (eNOS) is regulating TERT expression in the endothelium and impacts on cell aging (27, 28).

Taken together, the impact of physical activity on telomere biology is still not described sufficiently and in part controversial. Nevertheless, the latest research suggests that physical activity contributes to beneficial effects on telomeres due to various physiological mechanisms ensuring genetic stability and preventing from cellular senescence.

1.3 Aging and further investigated Factors

1.3.1 Aging and Telomere Biology

At all times, the humans have wanted to sustain youth and health (29). Many cultures have been developing diverse techniques and habits. For example in ancient Rome, Cicero has given different advice to cope with the phenomenon age (30). In our days, many processes have been discovered that could keep the body and mind healthy. For example, a study of calorie-restricted rodents has been showing that this restriction leads to longer vitality (31). Further, many studies are performed searching for the mechanism and markers of healthy aging (32).

Aging is a biological and wide-ranging process that is influenced by degenerative changes and damages induced by molecular pathways. Further, age is one of the major risk factors for many diseases including cardiovascular diseases, cancer, diabetes and neurological diseases. The suspected mechanism influencing the aging process are multifarious. Figure 4 gives an overview of some conceivable determinants of aging. Contributing factors may be DNA damage by oxidative stress, epigenetic modifications of genes, expression of regulatory non-coding RNA species and telomere shortening. Additionally, the current state of knowledge consists of multifaceted interactions between lifestyle and molecular systems. Apart from DNA damage including nuclear architecture defects and mitochondrial genome constancy, telomere attrition seems to play an important role. Further, a physiological decrease of telomere function that leads to aging and some pathological mechanisms on telomeres are observed that accelerate aging. For instance, research showed that the enzyme SIRT6 diminishes longevity in rodents. Additionally, it is suggested that disturbed homeostasis of proteins plays an

important role in aging due to the accumulation of misfolded proteins contributing to cellular senescence. More and more, mitochondria are in the focus of research. Some investigations discover accelerated aging caused by dysfunctional mitochondria. The processes behind stay unclear. Also, a scarce proliferation of stem and progenitor cells seems to be disadvantageous for the maintenance of the organism. Contrary, an extreme proliferation of these cells may lead to a damaging effect by quickening stem cell exhaustion. Further, inflammation influences the cell aging in diverse pathways. For instance, the inflammatory cytokine TGF-β inhibits telomerase gene expression. Moreover, aging also correlates with body configuration changes including reduced muscle mass, improved body fat and less organ mass except from the heart mass (5, 33-35).

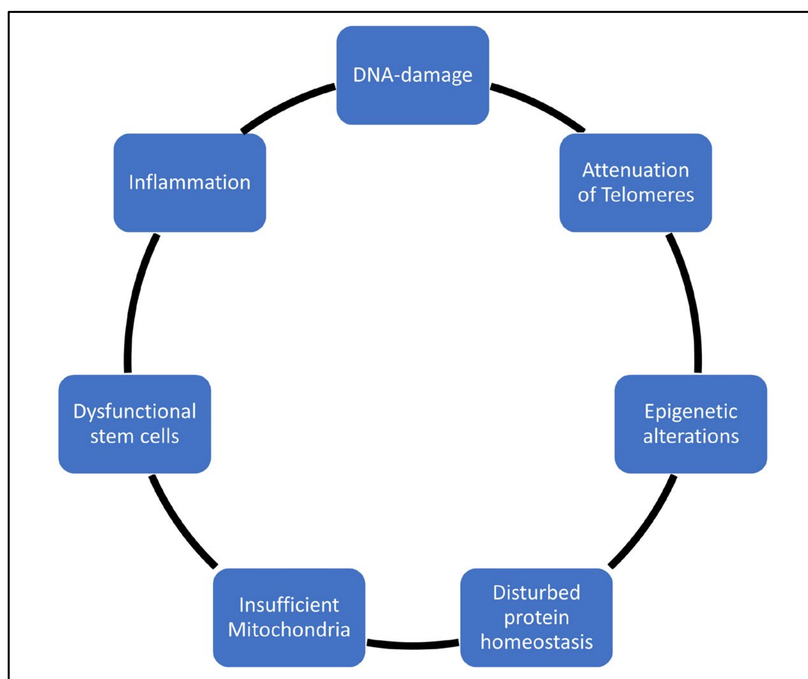

Figure 4: Conceivable Determinants of Aging
Source: own research

1.3.2 Obesity and Telomere Biology

Adiposity affects an increasing number of people worldwide. It is associated with numerous diseases like cardiovascular disease, neurodegeneration, osteoporosis and insulin resistance (36). Hence, more and more surveys are performed to investigate how obesity and aging are linked together. Lee et al. show in a longitudinal study an inverse association between higher total and abdominal adiposity and telomere length. Moreover, a negative correlation with systolic blood pressure, apolipoprotein B, waist circumference and telomere length is explored. This negative connection is stronger among young participants than older (37). Further, a survey including women older than 50 years and overweight shows longer telomere length in participants who lost weight in comparison to those who gained weight while this investigation (26). The research group of our institute comes to know a significant connection between relative telomere length and certain body composition including increased nuchal and hip fat thickness (38). Further investigations report that losing weight can beneficially regulate telomere length (17). In the year 2015, a systematic review and meta-analysis have been done to investigate the association between obesity and telomere length. The following combinations of terms have been used in a literature analysis: “telomere” AND “overweight” OR “obesity” OR “adiposity”. Sixty-three matching studies have been found including 119.439 subjects. In thirty-nine studies a weak or moderate correlation between telomere length and obesity is observable (39). This association is inter alia derived by two potential mechanisms. The first one postulates the direct damaging effects of adipose tissue on telomeres meaning obesity-induced conditions like oxidative stress and inflammation (38). The second mechanism is the direct regulation of energy metabolism by RAP1. RAP1 knockout mice are developing early onset of obesity with an accumulation of abdominal fat. Furthermore, Rap1-deficient mice suffer from multiple metabolic abnormalities (38, 40). Taken together, there is substantial evidence for an association of obesity and telomere biology.

1.3.3 Nutrition and Telomere Biology

A further investigated relation in the area of lifestyle and aging is the nutrition and its impact on telomere biology or rather health benefits. It is shown in multiple studies that different dietary patterns like calorie restriction, Mediterranean diet or vegetarian diet may lead to an increased telomere lengthening. Especially the Mediterranean diet is well evaluated. The diet is based on vegetables, seasonal fruits, olive oil, nuts, seeds, whole grains, fish, low-fat meat and moderate alcohol consumption. It is proofed evidence-based that this nutrition has a positive impact on the lower occurrence of some chronic diseases and lower morbidity in total. Thus, longevity is improved through lower levels of oxidative stress and inflammation caused by lower levels of C-reactive protein, interleukin 6, tumor necrose factor α and nitrotyrosine. This effect is declared by anti-oxidative and anti-inflammatory pathways. Additionally, certain ingredients like vitamin C, E, D, folate, β-carotene, polyphenols, curcumin and omega-3 fatty acids seem to be anti-oxidative and anti-inflammatory. Contrary, the calorie restriction and vegetarian diet study situation presents fewer and more controversial results. Basically, nuts, fruits, legumes, fruit juice, dairy products and coffee demonstrably seem to have a positive impact on telomere length. In contrast, nourishment with red meat, processed meat, high sugar and alcohol intake is negatively associated with telomere length. Considering all together, there is a link between healthy nutrition and telomere biology which is not clear to date and has to be evaluated in more detail (32, 41).

1.4 Summary

Recapitulating, an essential aspect of common and premature aging is constituted by telomeric dysfunction. It may be promoted by lifestyle factors like regular physical activity and obesity. More and more, reports come to know that physical activity and the absence of adiposity maintain telomere function by increasing the expression of shelterin proteins and telomerase. Further, it is proposed that telomere attrition may be caused by inflammatory reactions and anti-oxidative stress. However, the current stage of knowledge concludes that the causal molecular mechanisms remain largely

speculative. Correspondingly, potential interactions between telomere function, obesity and physical activity are currently unknown due to the lack of robust data. The results of such investigations could be applied for further studies using telomere length and shelterin proteins as biomarkers to design and monitor personalised exercise programs for primary and secondary disease prevention.

The findings of the planned study could be utilised for several purposes. On one side, the positive effect of physical activity on chronic diseases could be useful for rehabilitation issues and prescribed exercise interventions. The benefit for the patients could be validly evaluated and the costs get paid by the health care system. On the other side, the findings could be used for prevention issues. If there is a guaranteed association between telomere activity and physical activity habits, this can be used as a biomarker to predict age-related and chronic diseases.

2 Hypothesis

We hypothesise that physical activity prevents telomere shortening due to the expression of protective shelterin proteins.

3 Aims

The target of this present study is to determine the impact of sports on the cell aging process. For this purpose, we investigate the relative telomere length (RTL) and the expression of the shelterin complex due to the TERF-1 analysis in a preliminary subset of the TELOFIT study.

4 Methods

4.1 Study Cohort and Design

4.1.1 Inclusion Criteria

The study population consisted of middle-aged normal trained persons between 45 and 65 years without high adiposity or cachexia. The sportiness was defined by moderate leisure-time exercises up to 120 minutes per week.

4.1.2 Exclusion Criteria

Participants with the following criteria were excluded:

- Chronic diseases, especially cardiovascular diseases
- Pregnancy
- Obesity (BMI ≥ 30 kg/m²)
- Polypharmacy (more or equal than five drugs)
- high physical activity (more than 120 minutes high activity training per week)
- No performance of a leisure-time physical activity

4.1.3 Study Design and Intervention

Before the start of the intervention, a health status including an interview and physical examination was assessed by a physician. This involved the auscultation of the heart and lungs. Further, a check of the abdomen and neurological status was obtained. Furthermore, weight, height, blood pressure and heart rate were also surveyed. Additionally, a full blood analysis of laboratory routine parameters was performed.

Participants solved three blocks of exercise at low (40% of P_{max}), middle (55% of P_{max}) and high (70% of P_{max}) intensity. The three standardised interventions were

randomised in their order. Blood samples were obtained by the cubital puncture of the vein. Blood pressure was measured before the first exercise session and before the first of the five serial sessions. In summary, the subjects participated about three months at the program. The maximal power was calculated for each patient personally following the formula in figure 5 (42).

♂ $$P_{max} = (3 - \left(\frac{3 * (age - 30)}{100}\right) * weight$$

♀ $$P_{max} = (2{,}5 - \left(\frac{2{,}5 * (age - 30)}{100}\right) * weight$$

Figure 5: Calculation of Maximum Power
Source: Kindermann (41)

Every block was structured equally. At first, the intervention started with a single 30 minutes bicycle ergometer session including a blood collection before the intervention at day one. In the following four days of rest, there were also two blood draws executed after 24 and 48 hours. Then, five additional exercise sessions with the same intensity and duration in five consecutive days were performed in the next week. Further in these sessions, blood collections were obtained before the first of these five units. A third and fourth blood collection was taken before the second and fifth exercise. Finally, a blood draw was done three days later. Between the blocks, there was a break of at least eleven days and subsequently, one of the lacking blocks began. The procedure is shown schematically in figure 6.

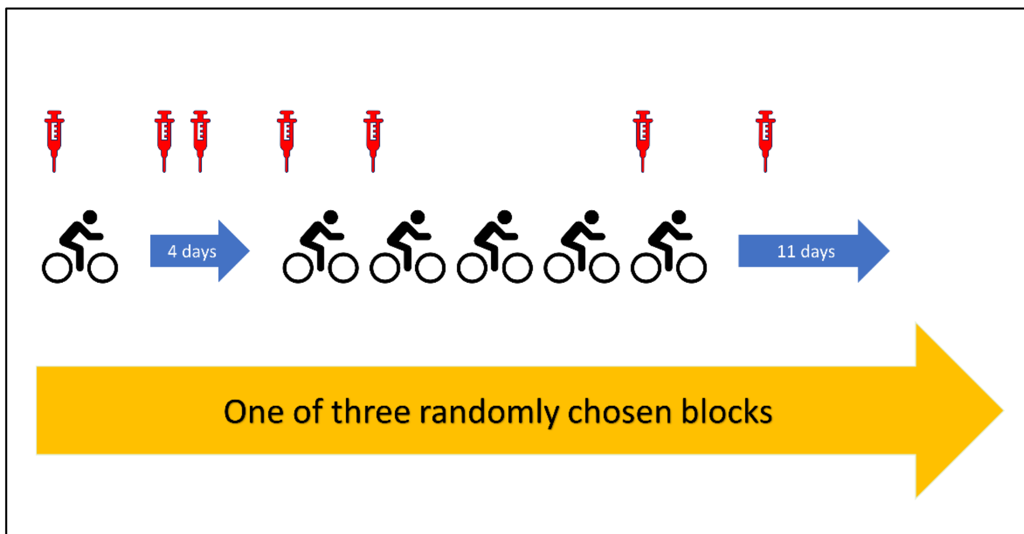

Figure 6: Process of a standardised Intervention Unit
: blood collection; : ergometer intervention (30 mins)

4.2 Ethics

The study was approved by the Ethics Committee of the Medical University Graz (EK-Nr. 30-168 ex 17/18).

The probands gave their informed consent by getting all necessary information and typical risk factors like phlebitis and heart attack cleared in the informed consent discussion. All probands taking part in the study were anonymised by coding with identification numbers.

4.3 Analytics

4.3.1 Blood Collection and Sample Preparation

We performed seven serial blood collections during each exercise block by venous puncture. Therefore, two different samples which are described in the following were analysed.

I. Firstly, the samples for protein extraction and TERF-1 quantification were drawn in Vacutainer CPT tubes (Becton Dickinson GmbH). This special kind of tubes was used to separate mononuclear cells (PBMCs) from the other components by a density gradient generated by Ficoll. For testing, we isolated and pelletised lymphocytes and monocytes. Subsequently, the samples were lysed for 30 mins on ice in a prepared 60 µl CHAPS buffer (100mM Tris-HCl (pH 7.4), 220mM NaCl, 10mM EDTA and 2% CHAPS) under non-denaturing conditions.
   Afterward, the samples were centrifuged and the supernatant was pipetted in 3 aliquots of 20 µl each. Subsequently, the samples were stored at -80°C.

II. Secondly, the samples for the quantification of relative telomere length were drawn in common EDTA tubes. Afterward, DNA isolation was automatically done with the MagNA Pure LC instrument (Roche) using the Total Nucleic Isolation Kit (Roche; Cat. No. 3 038 505). The isolated DNA was stored at -20°C.

4.3.2 Quantification of TERF-1

In order to quantify the TERF-1 protein level, we used a method based upon a sandwich enzyme-linked immune-sorbent assay (Human TERF1 ELISA Kit; Cat. No. EH12896; FineTest) as recommended by the manufacture. The 96-well plates were pre-coated with anti-TERF-1 antibodies, on which the TERF-1 of our samples binds. First, manufacture provided standards for the standard curve, test samples and biotin conjugated detection antibodies were applied in the wells. Subsequently, the excess was washed out with a wash buffer. Then, HRP Streptavidin was added and again, unbound conjugates were washed away. To start the HRP enzymatic reaction, the TMB substrate was added. This substrate was catalysed by HRP Streptavidin which resulted in blue colour. The adding of the acidic stop solution caused another colour reaction, changing the colour into yellow. The colorimetric reaction could be determined and quantified by the measurement of O.D. absorbance at 450nm using a micro-plate reader. The amount of TERF-1 protein is proportional to the density of the yellow colouring and its concentration in ng/ml can be interpolated from the standard curve.

In order to measure the total protein level for normalisation of the various samples, the Pierce™ BCA Protein Assay Kit (Thermo Scientific™; Cat. No. 23227) was used. It includes a detergent-compatible formulation based on bicinchoninic acid (BCA). The product of this assay ends up with a purple-coloured reaction. The total protein level is proportional to the intensity of the colorimetric reaction that can be determined and quantified by an O.D. absorbance at 562nm.

The concentration of TERF-1 - determined by ELISA as described above - was normalised to the total protein concentration for each sample to guarantee the comparability of samples.

4.3.3 Quantification of Relative Telomere Length

Relative telomere length in peripheral leucocytes was measured by a quantitative real-time PCR mainly developed by Cawthon using the PerfeCTa qPCR SuperMix Assay (Quantabio) (23). Minor modifications regarding the primers were implemented by O´Callaghan and were obtained from Metabio (43). The assay quantified the ratio of average telomere length (T) to a single-copy gene (S). The single-copy gene 36B4 was used as an amplification control for each sample and to determine genome copies per sample. All qPCR analyses were performed on the Thermocycler CFX96 Touch™ (Bio-Rad Laboratories) using SyberGREEN (Lonza). Each run included a standard curve made by dilutions of isolated and pooled DNA from multiple individuals to determine the quantity of the targeted templates. Relative telomere length was calculated as the ratio of telomere quantity to single-copy gene quantity (T/S ratio).

4.4 Statistics

Data are presented as means ± standard deviations. Continuous variables were compared using students t-test for dependent samples or Wilcoxon signed-rank test depending on the distribution of data. Correlations between variables were determined by linear regression analysis according to Pearson (r, Pearson

correlation coefficient). P-values <0,01 were considered statistically significant. Analyses were performed by explorative data analyses using SPSS-Package No. 26 for Windows (SPSS Inc., USA).

5 Results

5.1 Study Cohort

In this study, a preliminary subset of the TELOFIT study was investigated. The cohort started with seven test subjects. After two premature discontinuations, the cohort composed of five healthy middle-aged females. The probands in the present study were comparable in their health and lifestyle status, including age and body mass index.

One participant suffered from a mild hypertension, which was medically adjusted by a low dose antihypertensive and no hypertension had been detectable. Further, the determined clinical routine blood parameters of all five participants were without any diagnostic findings.

5.2 Baseline Characteristics

The mean age of the participants was 52 years with a standard deviation (SD) of ± 2,6 years. Concerning the body mass index, a mean of 24,6 kg/m^2 with an SD of ± 3,36 was measured. The blood pressure before each block was assessed with a mean systolic value of 116 mmHg, SD of ± 7,45 mmHg and a mean diastolic value 77 mmHg, SD of ± 5,91 mmHg. Further, the heart rate was measured with a mean of 73 bpm and an SD of ± 10,15 bpm. The mean height was 1,58 m and the SD was ± 0,04 m. Further, the mean and standard deviation of weight was obtained with 61,4 kg ± 9,31 kg (See table 1).

Table 1: Baseline Characteristics

	mean	**median**	**min**	**max**	**SD**
age (years)	52	51	50	57	2
bmi (kg/m^2)	24,6	23,5	20,2	29,9	3,3
bp diastolic (mmHg)	77	79	70	85	5
bp systolic (mmHg)	116	113	110	130	7
heart rate (bpm)	73	70	60	91	10
height (m)	1,58	1,57	1,53	1,64	0,04
weight (kg)	61,4	59,9	47,3	71,9	9,3

In this table following parameters are shown age (years), bmi (kg/m^2), bp diastolic (mmHg), bp systolic (mmHg), heart rate (bpm), height (m) & weight (kg)

5.3 Effects of Sports on RTL and TERF-1

To investigate the effects of physical activity, RTL and TERF-1 were determined at the beginning and the end of the sports program.

Figures 7 and 8 show the relative telomere length absolutely and relatively in the course of the three-month intervention per participant. However, the mean telomere length either stagnated or raised over the invention period and no statistically significant differences were detectable.

Figures 9 and 10 show the TERF-1 protein concentration absolutely and relatively in the course of the three-month intervention. It was a bride range from no to a high concentration increase of TERF-1 notable. However, no statistically significant differences were detectable.

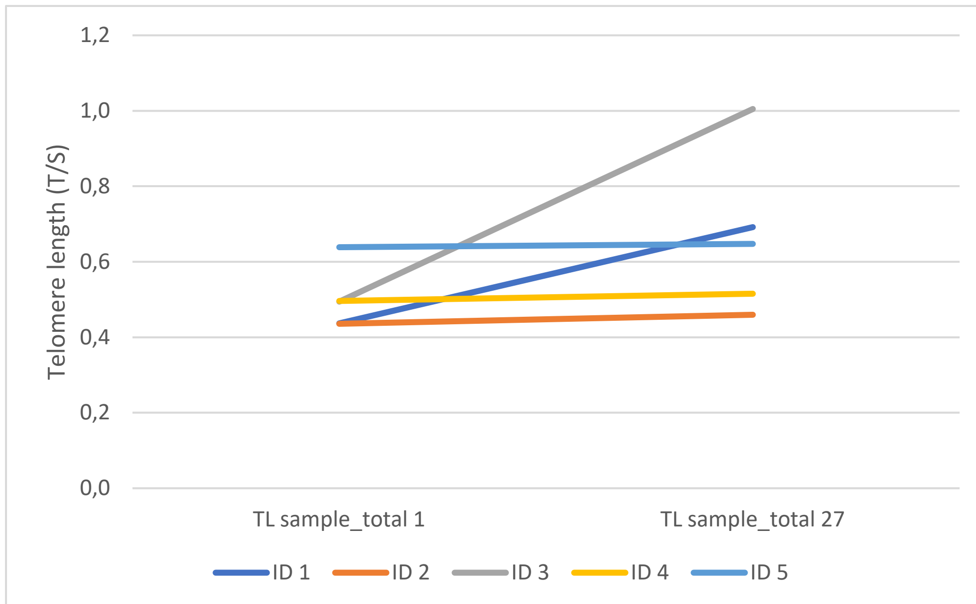

Figure 7: Measurement of RTL over the Intervention (absolute)
The chart shows the first and last measuring points of the relative telomere length in comparison; Every ID represents one participant; RTL is given as the T/S ratio

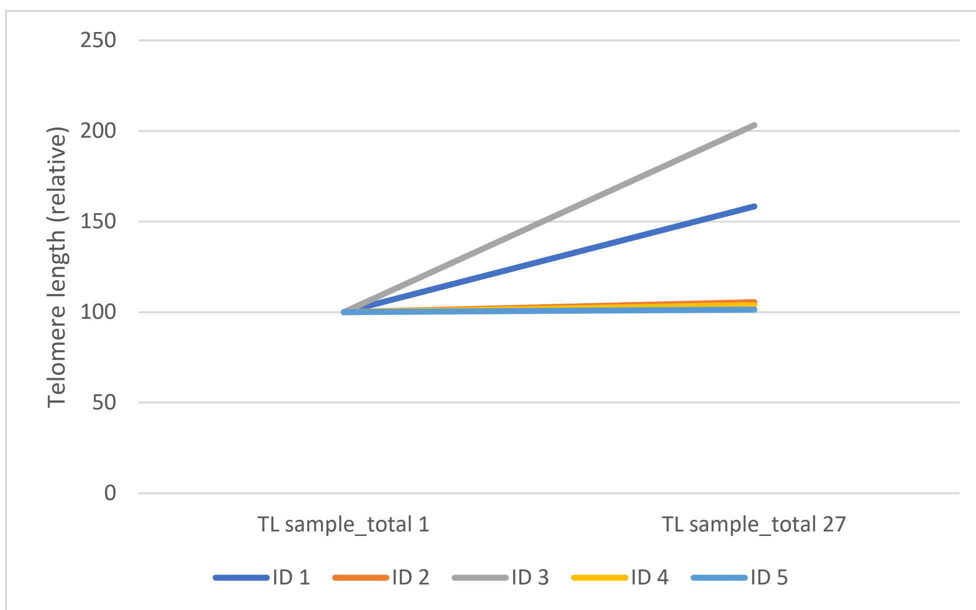

Figure 8: Measurement of RTL over the Intervention (relative)
The chart shows the first and last measuring points of the relative telomere length in comparison, normalizing the starting point to 100 percent; Every ID represents one participant

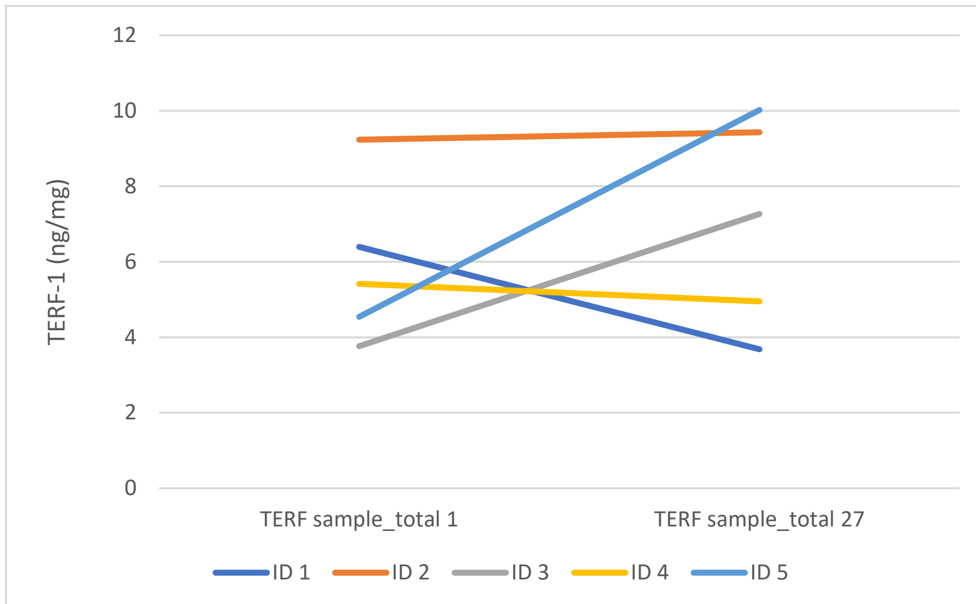

Figure 9: Measurement of TERF-1 over the Intervention (absolute)
The chart shows the first and last measuring point of the telomeric binding factor 1 in comparison; Every ID represents one participant; TERF-1 is given ng/mg

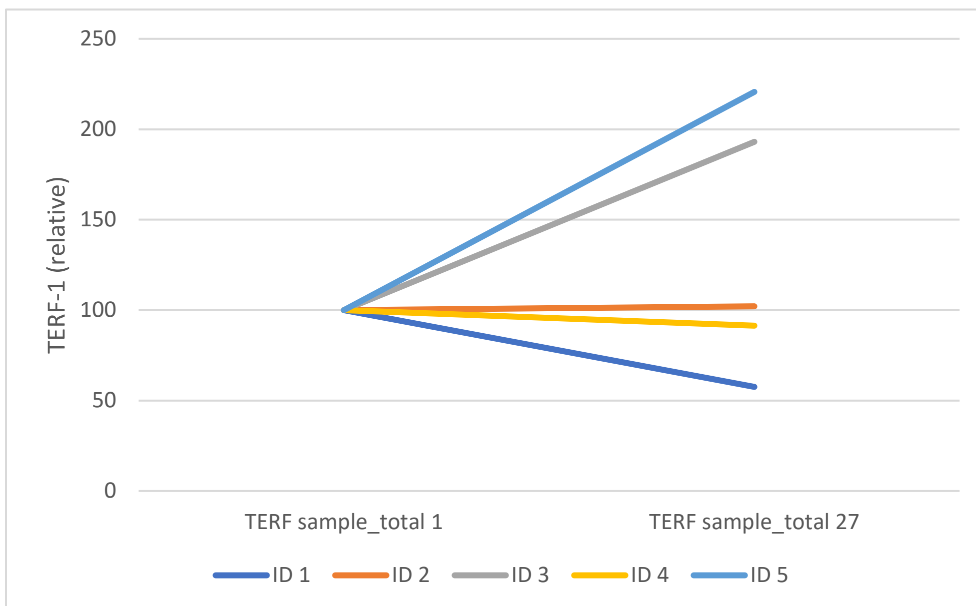

Figure 10: Measurement of TERF-1 over the Intervention (relative)
The chart shows the first and last measuring points of the telomeric binding factor 1 in comparison, setting the initial measurement to 100 percent; Every ID represents one participant

5.4 Impact of Training Intensity on Telomere Length

In figure 11 the three exercise levels (low, moderate and high) are compared to the mean telomere length for each block. The graphic shows a slight u-shape. It is visible that in low and high exercise blocks the mean telomere length is higher than in the moderate group (Figure11). Although, the differences did not reach statistical significance.

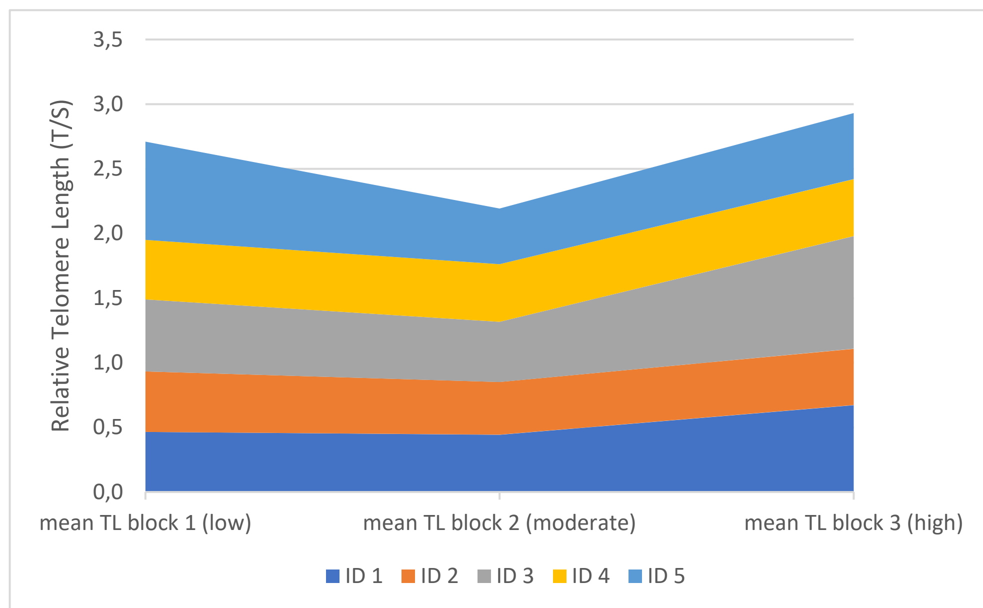

Figure 11: Mean Telomere Length Measurement per Intervention Block
The chart shows the mean relative telomere length accumulated per intensity block; Every ID represents one participant; RTL is given as the T/S ratio

5.5 Kinetics of TERF-1 Expression

Figure 12 presents TERF-1 expression during the three months intervention, averaged over all three intensity blocks. Regarding all seven measurement points, no collective tendencies were remarkable. Further, statistical analyses did not reveal any statistical differences in TERF-1 expression over the entire training program.

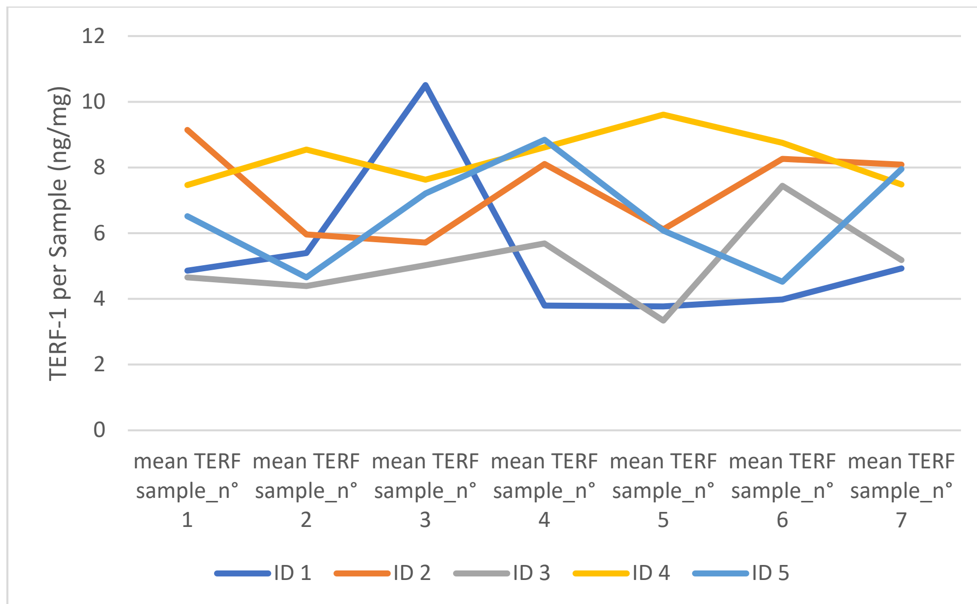

Figure 12: Mean TERF-1 Measurement per Sample
The chart shows the mean TERF-1 expression averaged per each point of measurement and per each participant; Every ID represents one participant; TERF-1 is given in ng/mg

5.6 Correlation between RTL and TERF-1 Expression

To determine if RTL is associated with the expression of the shelterin protein TERF-1, a correlation analysis was performed. In figure 13 the correlation analysis between relative telomere length and telomeric repeat binding factor 1 is illustrated. The correlation analysis by Pearson showed a highly significant inverse correlation (r =−0,49; p<0,01)

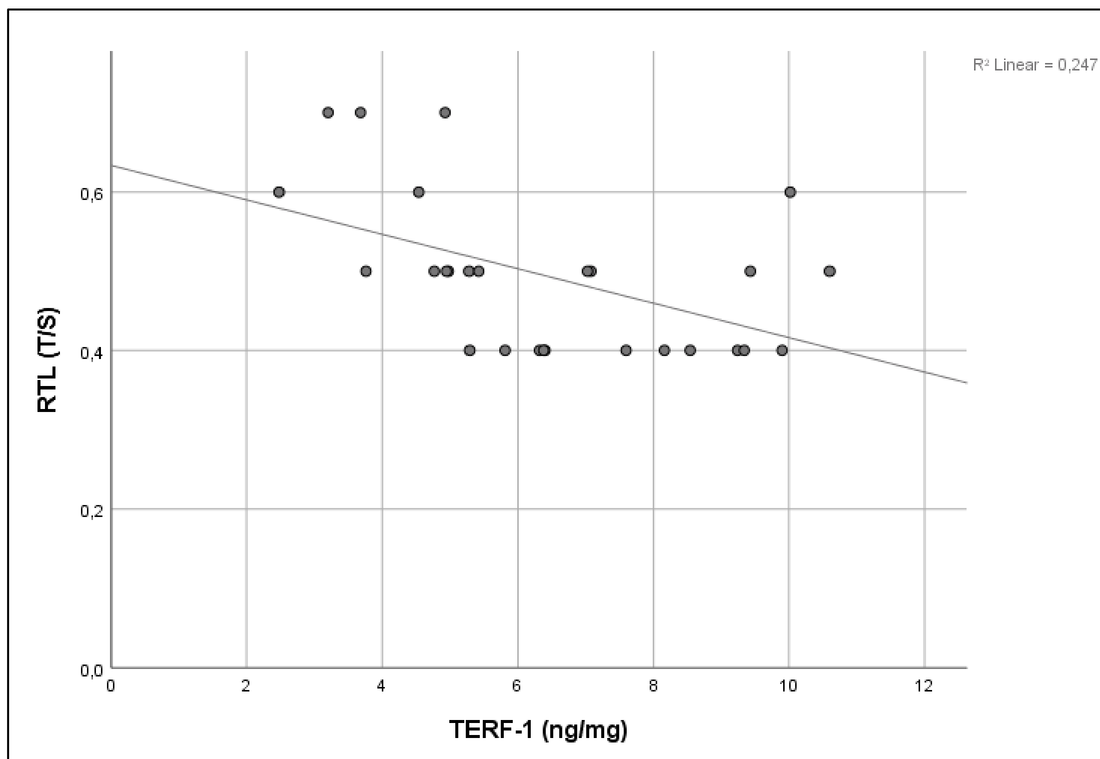

Figure 13: Regression Analysis of RTL and TERF-1
This diagram presents the correlation between RTL and TERF-1 within all measurements

6 Discussion

The findings of this study should be interpreted regarding the fact that underlying data are preliminary. In the period over the three months of intervention, we observed certain tendencies. In the case of the mean telomere length, a stagnating to increasing trend regarding the first measuring points compared to the last ones can be observed. An increasing tendency of telomere length caused by regular physical activity is in line with other studies (26, 44-47).

The measurements of the TERF-1 did not show any tendency to stagnate or increase. Herby, the expression of TERF-1 over the whole intervention varies in a wide range comparing the first point of measurement with the last one in each participant. Moreover, the analysis of TERF-1 expression over the entire training program for each participant did not show any statistically significant effects. Recent research also shows no clear effects of physical activity on the expression of shelterin proteins, determined by TERF-2 expression (48). However, it was shown in an experimental investigation that regular exercise at high intensity leads to lower TERF-1 expression in rodents (49).

Analysis of the impact of training intensity on telomere length indicated a less developed “U” shape in this study. One may speculate that lower and higher physical activity intensities relate to longer telomeres compared to moderate-intensity exercise. This finding contrasts with other studies that observed an inverted “U” shape (50, 51). These investigations showed that moderate physical activity is associated with the longest telomere length in comparison to low and high levels of activity.

Regressions analysis of relative telomere length and TERF-1 expression showed a highly significant inverse association ($r=-0,46$; $p<0,01$), indicating that the expression of the shelterin protein TERF-1 corresponds to shorter relative telomere length. Our finding is in line with current studies (52). However, the underlying mechanisms of this effect are not clear to date and discussed controversially.

6.1 Study Limitations

Interpretation of our findings must be done with caution concerning the small study population. The given preliminary dataset includes only 5 women and consequently, a selection bias must be estimated. This kind of recall problem is reported in the current literature (44, 53).

6.2 Outlook

Concerning the study design, a sedentary control group or further measurements with the same participants after a longer training period could be reconsidered. Here, a comparison between exercising and non-exercising individuals could be concluded. Naturally, a higher number of participants is needed and could emerge the validity of the results. Moreover, the defined points of measurement could be re-evaluated. For example, a measurement directly after an ergometer intervention could lead to a better understanding of how fast TERF-1 expression is changing. However, the ideal point of measurement is still discussed in the current literature (54). Additionally, a longer period of observation could lead to more reliable data and informative value could emerge. Moreover, an increased data set and long-term specific developments would be observable. Furthermore, it could be reassessed if the measurement of telomerase activity would be useful to understand the association between physical activity and telomere biology (45, 54, 55). Additionally, a point of discussion is the reliability of leucocyte telomere length as a suitable biomarker. For instance, muscle biopsies were used in some investigations to determine telomere length alterations instead of leucocytes extracted from the blood (53, 56, 57).

6.3 Conclusion

In conclusion, the present investigation demonstrates that physical activity with various intensities over three months shows no effects on relative telomere length or TERF-1 expression. It has to be reconsidered, beyond the small number of participants, that potential associations between relative telomere length and physical activity could be influenced by confounding, unmeasured or unknown aspects like undetectable genetic modifications or additional leisure time sports. In summary, further research with enlarged study cohorts is required to clarify the influencing factors and to highlight possible relations.

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Appendix

STUDY	PARTICIPANTS	PHYSICAL ACTIVITY / INTERVENTION	METHOD	RESULTS
CHERKAS, HUNKIN (51) (2008)	2401 (twins); F + M; 48,8 ± 12,9a	Self-reported PA	Southern blot	Sig. association between longer TL and higher levels of total PA
DIMAURO, SGURA (58) (2017)	24 (12 T2D + 12 C); M; ~62a	Intervention: 3x/week of 30 mins. moderate aerobic activity and 30 min calisthenics for 1 year	Q-FISH	Sig. difference in TL between all groups except trained T2D and trained control groups
FRETTS, METE (24) (2018)	2312; F + M; 40,3 ± 16,7a	Reported steps by pedometer	qPCR	Participants with more steps per day have longer TL than participants with fewer steps

FRIEDENREICH, WANG (59) (2018)	212 (99 E + 113 C); F; ~60a	Intervention: exercise >45 min, 5 d/week for 12 months	qPCR	No sig. difference of TL between exercise group and control group
KIM, KO (26) (2012)	44 (23 E + 21 C); postmenopausal F; 57,4 ± 5a	Exercise >60 mins., >3x/week, >12 months	qPCR	Longer TL in exercise group vs. sedentary control group
LAROCCA, SEALS (60) (2010)	25 (10 E + 15 C); F + M; 22 ± 1a & 32 (17 E + 15 C); F + M; 63 ± 2a	Vigorous aerobic exercise ≥5 days/week, >45 mins./day, ≥5 years	Southern blot	Older athletes have sig. longer TL than older sedentary
MATHUR, ARDESTANI (61) (2013)	32 (17 E + 15 C); F + M; 54 ± 4a	Marathon runner; 21 miles/ week, >5 years	FISH	No sig. difference of TL between runners and control group

OSTHUS, SGURA (53) (2012)	10 (5 E + 5 C); M; ~24a & 10 (5 E + 5 C); M; ~69a	Athletes vs. medium PA group	qPCR	Older athletes have sig. longer TL than older control group; between younger groups are no differences
PUTERMAN, WEISS (45) (2018)	68 (34 E + 34 C) F + M;	Exercise intervention vs. control group for 24 weeks	qPCR	Telomerase has no sig. difference; TL in exercise group is sig. longer than in control group
RAE, VIGNAUD (57) (2010)	37 (18 E + 19 C); F + M; ~40a	Almost 50,000 km training for >15 years	Southern blot	No sig. difference in TL between athletes and sedentary control group
SANFT, USISKIN (62) (2018)	125 (72 E + 53 C); Breast cancer survivor F; 57,8 ± 7,7a	Intervention: several 30 min. sessions/week and calorie reduction to 1200–2000 kcal/d for 6 months	qPCR	Probands with breast cancer (stage 0/I). TL in the intervention group is sig. longer than in the control group

SHADYAB, LAMONTE (44) (2017)	1405; F; 79,2 ± 6,7a	Accelerometer-measured light PA, moderate-to-vigorous PA and total PA for 1 week	Southern blot	TL is longer among women with ≥2.5 hours/week of MVPA than with <2.5 hours/week of MVPA
SHADYAB, LAMONTE (63) (2017)	1476; F; 79,2 ± 6,7a	Women's Health Initiative Physical Activity Questionnaire	Southern blot	Sig. longer TL for higher levels of total PA
SILVA, DE ARAUJO (64) (2016)	46 (15 intensive E + 16 moderate E + 15 C); M; 65–85a	Intensive training: ≥5 days/week; moderate training: 2-3 days/week	FlowFish	Athletes have sig. longer TL than untrained control group
SIMOES, SOUSA (47) (2017)	21 (11 E + 10 C); M; ~48a	>10 years of regular and competitive practice in sprint events	qPCR	Exercisers have sig. longer TL than the control group

SOARES-MIRANDA, IMAMURA (65) (2015)	582; F + M; 73 ± 5a	Minnesota Leisure-Time Activities questionnaire	Southern blot	Cross-sectional and longitudinal studies show no sig. link between PA and TL
SONG, VON FIGURA (66) (2010)	80 adults	Self-reported PA	qPCR	TL is not sig. correlated with PA
TUCKER (25) (2017)	5823; F + M; 46,3 ± 0,4a	MET	qPCR	High PA has sig. longer TL than adults in the low, moderate or sedentary groups
VENTURELLI, MORGAN (56) (2014)	24 (12 E + 12 C); F + M; ~ 87,5a & 12; F + M; ~25a	12 old– mobile, 12 old– immobile & 12 young	qPCR	Mobile older subjects have attenuated oxidative stress and age-related telomere shortening than old immobile subjects

VON KANEL, BRUWER (67) (2017)	203; F + M; 49,77 ± 8,67a	MET with ECG and activity recorder	qPCR	PA is not associated with TL
WERNER, FURSTER (28) (2009)	58 (32 E + 26C); F + M; ~21 & 46 (25 E + 21C) F + M; ~51a	Log-distance runners vs. healthy control group (young and old)	FISH & qPCR	Longer TL in athletes than non-athletes; Equal TL in young athletes and young non-athletes.
WERNER, HECKSTEDEN (46) (2019)	124 (26 aerobic E + 29 interval E + 34 resistance E + 35C); F + M; ~ 50a	Intervention for 6 months: exercise groups 3x/week; Control group maintain lifestyle	Flow Cytometry, FlowFish & qPCR	Aerobic & interval group show sig. longer TL; No lengthening in resistance & control group

Table 2: Meta-analysis of Studies investigating PA and TL

The table shows recent research investigating the influence of physical activity on telomere length. Age, a; Control group, C; Exercise group, E; Female, F; Male, M; Metabolic equivalents of task, MET; Minutes, mins; Physical activity, PA; Significant, sig.; Type 2 Diabetes, T2D; telomere length, TL